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CHARACTERIZATION OF *TOXOPLASMA GONDII* ISOLATES IN FREE-RANGE CHICKENS FROM AMAZON, BRAZIL

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ABSTRACT: The prevalence of *Toxoplasma gondii* in free-ranging chickens is a good indicator of the prevalence of *T. gondii* oocysts in the soil because chickens feed from the ground. The prevalence of *T. gondii* in 50 free-range chickens (*Gallus domesticus*) from Amazon, Brazil, was determined. Antibodies to *T. gondii* were assayed by the modified agglutination test (MAT) and found in 33 (66%) chickens with titers of 1:5 in 3, 1:10 in 2, 1:20 in 1, 1:40 in 1, 1:80 in 2, 1:160 in 5, 1:200 in 9, 1:400 in 5, 1:800 in 2, 1:1,600 in 2, and 1:3,200 or higher in 1. Hearts and brains of 33 seropositive chickens were bioassayed individually in mice. Tissues from 17 seronegative chickens were pooled and fed to 2 *T. gondii*–free cats. Feces of cats were examined for oocysts, but none was found. *Toxoplasma gondii* was isolated from 24 chickens with MAT titers of 1:5 or higher. Genotyping of these 24 *T. gondii* isolates by polymorphisms at the SAG2 locus indicated that 14 were type I, and 10 were type III; the absence of type II strains from Brazil was confirmed. Fifty percent of the infected mice died of toxoplasmosis, irrespective of the genotype.

Toxoplasma gondii infections are widely prevalent in human beings and other animals worldwide (Dubey and Beattie, 1988). Humans become infected postnatally by ingesting tissue cysts from undercooked meat, consuming food or drink contaminated with oocysts, or by accidentally ingesting oocysts from the environment. However, only a small percentage of exposed adult humans develop clinical signs. It is unknown whether the severity of toxoplasmosis in immunocompetent persons is because of the parasite strain, host variability, or other factors.

Toxoplasma gondii isolates have been classified into 3 genetic types (I, II, III) on the basis of restriction fragment length polymorphism (RFLP; Howe and Sibley, 1995; Howe et al., 1997; Mondragon et al., 1998; Owen and Trees, 1999; Fuentes et al., 2001; Grigg et al., 2001; Ajzenberg et al., 2002; Boothroyd and Grigg, 2002; Jungersen et al., 2002; Aspinall et al., 2003; Ajzenberg et al., 2004; da Silva et al., 2005). The parasite used to be considered clonal, with very low genetic variability. However, most of the information was derived from isolates from Europe and North America. On the basis of newer markers for genetic characterization and with the use of recently isolated strains from Brazil and French Guiana, a higher genetic variability has been revealed than previously reported (Ajzenberg et al., 2004; Lehmann et al., 2004).

We have initiated a worldwide study of *T. gondii* population structure. For this purpose, we have chosen the free-range chicken as the host indicator for soil contamination with *T. gondii* oocysts because they feed from the ground. Thus far, we have characterized strains from South America (Brazil [Dubey et al., 2002; Dubey, Graham, da Silva et al., 2003; Dubey, Navarro et al., 2003], Peru [Dubey, Levy et al., 2004], Guatemala [Dubey, Lopez et al., 2005], Venezuela [Dubey, Lenhart et al., 2005], Argentina [Dubey, Venturini et al., 2003; Dubey, Marcet, and Lehmann, 2005], Colombia [Dubey, Gomez-Martin et al., 2005]), the Caribbean (Grenada, West Indies; Dubey, Bhaiyat et al., 2005), North America (United States [Dubey, Graham, Dahl, Sreekumar et al., 2003; Lehmann et al., 2003], Mexico [Dubey, Morales, and Lehmann, 2004]), Africa (Egypt [Dubey, Graham, Dahl, Hilali et al., 2003], Mali, Kenya, Burkina Faso, and Democratic Republic of Congo [Dubey, Karhemere et al., 2005)], Asia (Sri Lanka [Dubey, Rajapakse et al., 2005], India [Sreekumar et al., 2003], Israel [Dubey, Salant et al., 2004]), and Europe (Portugal [Dubey, Vianna et al., 2006], Austria [Dubey, Edelhofer et al., 2005]). These studies are still not complete, and genetic diversity has been examined in some detail only for the isolates from chickens from Brazil (Dubey et al., 2002; Dubey, Graham, Silva et al., 2003; Dubey, Navarro et al., 2003). Nevertheless, a pattern is emerging that indicates isolates from Brazil are genetically distinct (Lehmann et al., 2004).

In this study, we attempted to isolate *T. gondii* from chickens from Amazon, Brazil, which is geographically, socially, and ecologically different from other parts of the world. Additionally, the distribution of *T. gondii* in the heart and brain of chickens was compared.

MATERIALS AND METHODS

Naturally infected chickens

Chickens were obtained from Monte Negro County, Rondônia state, western Amazon, Brazil (10°15′35″S, 63°18′6″W). It has a population of approximately 13,000, mostly in rural areas on small, family farms. The region has a hot and humid climate, with high levels of precipitation that averages 2,000 mm annually, and a moderate drought period from April to October. Temperature ranges from 25 to 29 C with relative humidity of 70–80% throughout the year (Camargo et al., 2002).

The rural area of Monte Negro has approximately 800 farms, with most rearing free-roaming chickens for subsistence. Chickens (n = 50) were purchased from 11 of these farms during February 2005. These farms were selected because they also sell live chickens in the local urban food market on Sundays. The number of hens sampled from each farm ranged from 2 to 6. Chickens were slaughtered in the laboratory, where brain, heart, and blood samples were collected and kept at 4 C until sent cold by air to Beltsville, Maryland. Five to 6 days elapsed between killing of chickens and receipt of samples. Samples were received in excellent condition.

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TABLE I. Isolation of *Toxoplasma gondii* from seropositive chickens from the Amazon, Brazil.

			Isolation in mice†							
			Brain			Heart				
Chicken no.	Farm code, location	Chicken MAT*	No. infected	No. died	Day of death	No. infected	No. died	Day of death	Strain designation	Genotype
7	2, line 25	200	0	0	0	5	3	22, 24, 32	TgCkBr117	III
13	3, line 25	5	2	0	0	0	0	0	TgCkBr118	I
16	4, line 25	200	2	0	0	0	0	0	TgCkBr119	I
17	4, line 25	200	1	1	39	0	0	0	TgCkBr120	I
20	4, line 25	400	0	0	0	4	1	29	TgCkBr121	I
21	5, line 25	20	1	0	0	1	0	0	TgCkBr122	I
23	5, line 25	200	2	2	23, 40	3	3	14, 17, 20	TgCkBr123	I
27	6, line 30	160	5	5	15, 16, 16, 17, 20	4	4	18, 18, 20, 27	TgCkBr124	I
30	6, line 30	800	1	0	0	5	5	15, 17, 19, 19, 32	TgCkBr125	III
31	8, TB24	>3,200	1	1	29	5	5	14, 15, 16, 17, 22	TgCkBr126	III
32	8, TB24	160	0	0	0	5	5	19, 21, 22, 23, 28	TgCkBr127	III
33	8, TB24	400	0	0	0	4	4	16, 20, 20, 39	TgCkBr128	III
35	8, TB24	400	0	0	0	1	1	39	TgCkBr129	I
36	10, line 30	80	5	0	0	0	0	0	TgCkBr130	III
37	10, line 30	200	0	0	0	5	0	0	TgCkBr131	III
38	10, line 30	160	5	0	0	0	0	0	TgCkBr132	III
39	10, line 30	800	5	0	0	5	2	22, 43	TgCkBr133	III
40	10, line 30	400	0	0	0	1	0	0	TgCkBr134	III
41	11, line 30	200	1	0	0	0	0	0	TgCkBr135	I
42	11, line 30	1,600	1	0	0	4	4	17, 17, 17, 19	TgCkBr136	I
43	11, line 30	80	1	0	41	1	0	36	TgCkBr137	I
44	11, line 30	160	5	5	14, 15, 17, 18, 20	5	5	12, 13, 14, 17, 17	TgCkBr138	I
45	11, line 30	320	0	0	0	1	1	21	TgCkBr139	I
46	7, urban	5	1	1	41	0	0	0	TgCkBr140	I

* MAT, modified agglutination test.
† Five mice were inoculated per tissue sample.

Serological examination

Sera of chickens were tested for *T. gondii* antibodies with 2-fold serum dilutions from 1:5 to 1:3,200 by the modified agglutination test (MAT) as described by Dubey and Desmonts (1987).

Bioassay of chickens for *T. gondii* infection

Tissues of all chickens were bioassayed for *T. gondii* infection. Brains and hearts of 33 chickens with MAT titers of 1:5 or higher were each bioassayed individually in out-bred female Swiss Webster mice obtained from Taconic Farms, Germantown, New York, as described by Dubey et al. (2002). Each tissue was homogenized individually, digested in acidic pepsin, neutralized, and washed; the homogenate inoculated subcutaneously into 5 mice. In total, 10 mice were inoculated with tissues of each chicken.

Brains and hearts from 17 seronegative (MAT < 1:5) chickens were pooled and fed separately to 2 *T. gondii*–free cats (Dubey et al., 2002). Feces of cats were examined for shedding of *T. gondii* oocysts 3–14 days postingestion of chicken tissues as previously described (Dubey, 1995). Fecal floats were incubated for 1 wk at room temperature to allow sporulation of oocysts and were bioassayed in mice (Dubey and Beattie, 1988). Tissue imprints of mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on days 40–42 postinoculation (PI), and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies by MAT. Mice were killed 45–48 days PI, and brains of all mice were examined for tissue cysts as described (Dubey and Beattie, 1988). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues.

Genotyping and sequencing

Toxoplasma gondii DNA was extracted from the tissues of a single infected mouse from each group (Lehmann et al., 2000). The RFLP strain type of *T. gondii* isolates was determined by nested PCR on the SAG2 locus according to Howe et al. (1997).

RESULTS

Antibodies to *T. gondii* were found in 33 chickens with titers of 1:5 in 3, 1:10 in 2, 1:20 in 1, 1:40 in 1, 1:80 in 2, 1:160 in 5, 1:200 in 9, 1:400 in 5, 1:800 in 2, 1:1,600 in 2, and 1:3,200 or higher in 1.

Toxoplasma gondii was isolated from 24 chickens from 9 properties; from 2 chickens with titers of 1:5, from 2 with a titer of 1:20, from 1 with a titer of 1:80, and from 19 with titers of 1:160 or higher. It was isolated from the brains of 7, hearts of 8, and both brains and hearts of 9. *Toxoplasma gondii* isolates from 19 of 24 were lethal for mice; all mice that became infected with isolates from 3 chickens (nos. 27, 31, and 44) died of acute toxoplasmosis. Tachyzoites were found in lungs of all infected mice that died during the second and third week PI. Tissue cysts were easily found in the brains of mice that died or were killed the fourth week PI, except in a mouse that died day 36 PI after inoculation with the heart of chicken no. 43; in this mouse, there were numerous tachyzoites in the lung, but no tissue cysts in approximately one-half the brain examined by squash preparations. In 13 instances, only 1 of the 5 mice inoculated with infected chicken tissue was positive for *T. gondii*. These *T. gondii* isolates were designated as TgCkBr117–141 (Table I). The previous 98 chicken isolates from Brazil are designated in this paper as TgCkBr1–116. The São

TABLE II. Mouse mortality patterns of *Toxoplasma gondii* isolates from chickens from Brazil.

Source (reference)	No. of isolates	No. of isolates (% mortality)						Total no. of mice dead	Day of death					
		0	20–25	40–50	60	75–80	100		0–7	8–14	15–21	21–28	29–35	≥36
São Paulo State (Dubey et al., 2002)	22	2	1	0	0	0	19	68	0	32	30	6	0	0
Rio de Janeiro State (Dubey, Graham, da Silva et al., 2003)	67	7	2	5	2	1	50	269	1	55	156	54	3	0
Paraná (Dubey, Navarro et al., 2003)	13	3	2	0	0	0	9	42	0	12	26	3	1	0
Amazon (this study)	24	9	2	0	1	2	10	58	0	4	35	8	4	7
Total	126	21	5	5	3	3	88	437	1	103	247	71	8	7

Paulo State chicken isolates are designated as TgCkBr1–25 from chickens nos. 14, 16, 19, 21, 29, 32, 36, 39, 42–46, 52, 56, 57, 59, 61, 67, 68, 70, 73, pool A, pool B, and pool C chickens, respectively (Dubey et al., 2002). The Rio de Janeiro State chicken isolates are designated as TgCkBr26–92 from chickens nos. 3, 8–12, 14, 16–20, 23–26, 28, 29, 35–37, 42, 43, 45, 47, 48, 55–57, 59, 61, 63, 65, 66, 69–72, 74, 78, 83, 88, 89, 94, 97, 100–102, 182, 186, 188, 190–192, 194, 195, 202, 204–206, 210, 214, 218, 228–231, respectively (individual chickens were not designated by Dubey, Graham, da Silva et al., 2003). The Paraná chicken isolates are designated as TgCKBr93–106 from chickens nos. 2, 7, 8, 12, 13, 20, 23, 25, 34, 36, 38–40, and pool A, respectively (Dubey, Navarro et al., 2003). Genotyping of 24 isolates by SAG2 indicated that 14 were type I, and 10 were type III. Nearly 50% of infected mice died of toxoplasmosis, and this was not associated with the genotype (Table I).

The 2 cats fed tissues of seronegative chickens did not shed oocysts.

DISCUSSION

In this study, *T. gondii* was isolated from 24 of 33 (72.7%) seropositive chickens and not from seronegative chickens, supporting the specificity of the MAT. It is interesting that viable *T. gondii* was obtained from tissues of 2 chickens with titers of only 1:5; these sera were of good quality and without prozone. Titers of <1:25 are regarded as nonspecific, and sera are generally screened starting at 1:20 or 1:25 serum dilution in MAT (Dubey, Thulliez, and Powell, 1995; Dubey, Thulliez, Weigel et al., 1995; Dubey, Weigel et al., 1995). However, in any population there will be a few infected individuals with a low titer, and these will likely not be accounted for by a routine serological screen. In this study and others with chickens (see Dubey, Marcet, and Lehmann, 2005), data are being accumulated for the validity of MAT for the detection of *T. gondii* in chickens.

In this study, *T. gondii* was isolated from the brains and not hearts of 7 chickens from the Amazon. This finding is of interest because in our previous studies, *T. gondii* was localized more frequently in the heart than the brains of chickens (Dubey, Levy et al., 2004). Therefore, both brains and hearts should be used for the isolation of viable *T. gondii* from chickens. The success of isolation also depends on the number of mice and the amount of tissue bioassayed. In this study, entire brains and hearts were used to isolate *T. gondii*, and most of the tissue digest was inoculated into 5 mice. It is noteworthy that in 13 instances, only 1 of the 5 mice inoculated with tissue digests became infected with *T. gondii*, indicating that the concentration of *T. gondii* in tissues is low.

Before the discovery of genotyping (Howe and Sibley, 1995) *T. gondii* isolates were phenotypically classified as mouse virulent or avirulent. Genetic type I strains were mouse virulent, whereas type II and type III strains were avirulent or mildly virulent for mice. Type I strains killed all mice within 2 wk PI, irrespective of the dose. However, these data are based on isolates that have been adapted to mice and maintained in mice for an unknown time (Howe and Sibley, 1995). Very little data on mouse mortality are based on primary isolations. We have started to accumulate such data on the basis of isolates from chickens according to a specified protocol (subcutaneous inoculation of tissue digest into 5 Swiss Webster mice). Results from 126 isolates of *T. gondii* from chickens from Brazil are summarized in Table II. Most (83.3%) *T. gondii* isolates killed infected mice. Most (96.3%) infected mice died of toxoplasmic pneumonia between 10 and 28 days PI, except 1 mouse that died day 6 PI, probably because of bacterial infection (it was found autolyzed). Tachyzoites were found in smears of lungs of mice that died during the second and third week PI. Unlike *T. gondii* isolates from chickens from Israel (Dubey, Salant et al., 2004) and Austria (Dubey, Edelhofer et al., 2005), it was difficult to find tissue cysts, which were easily found in the brains of seropositive mice that were killed 6 wk PI with the chickens from Brazil.

Of the 110 isolates of *T. gondii* from chickens from Brazil so far genotyped, most (67%) were type I and none was type II. This is in marked contrast to isolates from North America (see Dubey, Marcet, and Lehmann, 2005).

The seroprevalence of *T. gondii* in humans in Brazil is high, particularly in the Amazon (Baruzzi, 1970; Leser et al., 1977; Lovelace et al., 1978; Souza et al., 1987; Glasner et al., 1992; Bahia-Oliveira et al., 2003; Sobral et al., 2005). The ingestion

of *T. gondii* oocysts is considered the main mode of transmission in Brazil because in economically deprived areas, people cannot afford to eat meat (Bahia-Oliveira et al., 2003; de Moura et al., in press) and in certain tribes do not eat red meat (Sobral et al., 2005). The results of this study provide the first evidence of the environmental contamination by *T. gondii* oocysts in Amazon because chickens feed from the ground.

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